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Der Präsident des Europäischen Patentamts;
Im Auftrag

For the President of the European Patent Office

Le Président de l'Office européen des brevets
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(Falls die Bezeichnung der Erfindung nicht angegeben ist, siehe Beschreibung.
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Use of EDG2 receptor in an animal model of heart failure

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Use of EDG2 receptor in an animal model of heart failure

The invention refers to a transient transformed mammal which is useful as animal model for heart failure.

G protein-coupled receptors (GPCRs) play a central role in a multiplicity of physiological processes. It is assumed that in the human genome about 1000 genes code for this receptor family. Approximately 60 % of the pharmaceuticals presently available through prescription act as GPCR agonists or antagonists. This underlines the importance of this receptor class for the pharmaceutical research industry. Owing to the size and importance of said protein family and in view of the fact that physiological ligands are still unknown for many GPCRs (orphan GPCRs), it can be assumed that this receptor class will be one of the most important reservoirs for suitable target proteins in the search for novel medicinal substances in the future.

GPCRs are a family of integral membrane proteins which are located on cell surfaces. They receive signals from extracellular signaling substances (e.g. hormones, neurotransmitters, peptides, lipids) and transfer these signals into the cell interior via a family of guanine nucleotide-binding proteins, the "G proteins". Depending on the receptor specificity, the G protein activated and the cell type, these receptors induce various signal transduction pathways.
All GPCR polypeptide chains fold into seven α-helices which span across the phospholipid bilayer of the cell membrane. The seven membrane passages result in the formation of extra- and intracellular loops which allow extracellular ligand binding and intracellular coupling of G proteins. For this reason, GPCRs are also denoted seven-pass transmembrane receptors.

All G protein-coupled receptors act according to a common basic pattern: binding of an extracellular ligand leads to a conformational change in the receptor protein which enables the receptor protein to contact a G protein. G protein-mediated signal transduction cascades in the cell finally lead to a biological response of the cell.

G proteins are heterotrimeric proteins which consist of the subunits α, β and γ. They are located on the inside of the cell membrane via lipid anchors. Coupling of activated GPCRs to G proteins induces a GDP/GTP exchange at the Gα subunit and dissociation of the heterotrimeric G protein into an α and a $\beta\gamma$ subunit. Both the activated α subunit and the $\beta\gamma$ complex are able to interact with intracellular effector proteins.

Activation of membrane-bound adenylate cyclase (AC) by Gαs-type G proteins, for example, leads to an increase in the intracellular cAMP level or, in the case of activation by Gαi-type G proteins, to the decrease therein. Gq-type G proteins activate phospholipase C (PLC) which catalyzes the formation of inositol 1,4,5-triphosphate (IP3) and diacylglycerol (DAG). These molecules lead to the release of Ca^{2+} from intracellular storage organelles or to activation of proteinkinase C (PKC).

The polynucleotide sequence and the amino acid sequence of the human EDG2 (Endothelial Differentiation Gene 2) has been made available to the public. The sequence is available for example from NCBI (Accession: NM_001401). The protein sequence is available from Swiss Prot (Accession: Q 92633). Cloning of the receptor from a human lung cDNA library was published in "An et al., Biochem. Biophys. Res. Commun. 24, 231 (1997)".

The full length sequence encodes a 359 amino acid protein which belongs to the superfamily of guanine nucleotide-binding protein-coupled receptors (GPCR). Human EDG2 mRNA is widely distributed in human tissues with the highest abundance in brain. HEK293 cells expressing the human EDG2 protein showed an elevated response to lysophosphatidic acid (LPA) in a serum response element reporter gene assay, which was LPA concentration dependent and specific to LPA. The mouse counterpart of EDG2 protein was also identified as a receptor for LPA.

Lysophosphatidic acid (LPA) and sphingosine 1-phosphate (S1P) are potent phospholipid mediators with diverse biological activities. Their appearance and functional properties suggest possible roles in development, wound healing, and tissue regeneration. The growth-stimulating and other complex biological activities of LPA and S1P are attributable in part to the activation of multiple G protein-mediated intracellular signaling pathways. Several heterotrimeric G proteins, as well as Ras-

and Rho-dependent pathways play central roles in the cellular responses to LPA and S1P.

Within the scope of this invention and in all cases used and without any exemption mammal shall not encompass the human species (Homosapiens) or an individual of Homosapiens of part of a body of a human.

The invention refers to a myocardial cell of a mammal which cell contains an adenoviral vector sequence for simultaneous expression of a G protein coupled receptor EDG2 and GFP (Green Fluorescent Protein).

The adenoviral vector sequence consists preferably of a recombinant E 1/E 3 deficient adenovirus which expresses the G protein coupled receptor EDG2 and GFP under control of two independent promoters. Such promoters could be two CMV promoters.

The myocardial cell of a mammal which contains an adenoviral vector sequence as aforementioned expresses the G protein coupled receptor EDG2 and GFP and contains therefore protein of the G protein coupled receptor EDG2 and protein of GFP.

The myocardial cell which contains an adenoviral vector sequence is preferably the cell of a rabbit, a mouse or a rat.

The invention refers also to production of a myocardial cell which cells contains an adenoviral vector sequence for simultaneous expression of G protein coupled receptor EDG2 and GFP wherein

a] the heart of a mammal is removed by state of the art veterinary medicine operative techniques,

b] the heart is perfused and digested with collagenase,

c] the isolated cardiomyocytes are infected with an adenoviral vector consisting of a recombinant E1/E3 deficient adenovirus which allows for expression of

the G protein coupled receptor EDG2 and GFP under control of two independent promoters. Such promoters are preferably two CMV promoters.

Furthermore the invention refers to a mammal having a myocardium which contains an adenoviral vector for simultaneous expression of a G protein coupled receptor EDG2 and GFP. This adenoviral vector sequence of the mammals consists preferably of a recombinant E1/E3 deficient adenovirus which allows for expression of the G protein coupled receptor EDG2 and GFP under control of two independent promoters. Such two independent promoters are preferably two CMV promoters.

The invention refers also to a mammal having a myocardium which contains a protein of G protein coupled receptor EDG2 and a protein of GFP. Such a mammal having a myocardium with an adenoviral vector for simultaneous expression of a G protein coupled receptor EDG2 and GFP and/or having a myocardium with protein of G protein coupled receptor EDG2 and protein of GFP is preferably a rabbit, a mouse, or a rat. Furthermore the invention refers to production of a mammal having a myocardium with an adenoviral vector for simultaneous expression of a G protein coupled receptor EDG2 and GFP and/or having a myocardium with protein of G protein coupled receptor EDG2 and protein of GFP wherein

a] an adenoviral vector sequence for simultaneous expression of G protein coupled receptor EDG2 and GFP is provided,

b] a mammal is provided,

c] the adenoviral vector system from a] is transferred into the myocardium of the mammal from b] by means of a catheter.

The invention concerns also use of a mammal having a myocardium with an adenoviral vector for simultaneous expression of a G protein coupled receptor EDG2 and GFP and/or having a myocardium with protein of G coupled receptor EDG2 and protein of GFP for producing myocardial cells which can be taken for a method for identification of a compound which modifies the activity of G protein coupled receptor EDG2.

The invention refers to a method for identification of a compound which modifies the activity of receptor EDG2 wherein

a] a transformed cell from a heart muscle which expresses the receptor EDG2 or a fusion protein comprising the receptor EDG2 is provided,

b] possibly a treatment of the cell from a] is performed by use of isoproterenol and/or lyophosphatidic acid,

c] a chemical compound is provided,

d] the cell from a] or b] is brought in contact with the chemical compound from c],

e] the contractility of a cell from d] is determined and is brought in relation to the contractility of a cell which has the same characteristics as a cell from a] but which has not brought in contact with a chemical compound from c] and wherein a relative enhancement or reduction of contractility of the cell which has brought in contact with a chemical compound according to d] by this compound demonstrates the ability of such compound to modify the activity of receptor EDG2.

The invention refers furthermore to a method for identification of a compound which modifies the activity of receptor EDG2, wherein

a] a transformed cell from a heart muscle which expresses the receptor EDG2 or a fusion protein comprising the receptor EDG2 is provided,

b] possibly a treatment of the cell from a] is performed by use of proterenol and/or lysophosphatidic acid,

c] a chemical compound is provided,

d] the cell from a] or b] is brought in contact with the chemical compound from c],

e] the contractility of a cell from d] is determined and is brought in relation to contractility of a cell of same cell type as a cell according to a] but which does not express a receptor EDG2 or a fusion protein comprising a receptor EDG2 wherein a relative enhancement or reduction of contractility of the cell which expresses a receptor EDG2 or a fusion protein comprising a receptor EDG2 by a compound demonstrates the ability of such compound to modify the activity of receptor EDG2.

The invention refers furthermore to an adenoviral vector consisting of one polynucleotide of the following groups:

a] a polynucleotide having a sequence as specified in SEQ ID NO. 5,

b] a polynucleotide which is 95 % identical to the polynucleotide of SEQ ID NO. 5,

c] a polynucleotide which is at least of the same length as the polynucleotide of SEQ ID NO. 5 and which hybridizes to a polynucleotide of SEQ ID NO. 5 when applying highly stringent hybridization conditions.

The adenoviral vector sequence encompasses preferably a polynucleotide sequence which is encoding a protein of SEQ ID NO. 2.

Hybridization means assembly of two single polynucleotide strains which have complementary sequences to double stands. Hybridization might occur between two DNA-strand, one DNA- and one RNA-strand as well as between two RNA-strands. Forming of hybrid polynucleotide strands may start from a solution which contains double stranded polynucleotide molecules by heating this solution to separate the double strands in single stranded polynucleotides. The heating step could consist of boiling in a water bath during 10 to 20 minutes. When the solution is slowly cooled down to room temperature after it was heated the hybridization to double stranded molecules will occur. Under experimental conditions the hybridization is commonly carried out by means of hybridization filters which polynucleotides have been fixed upon by blotting or electrophoresis. Hybridization might be visualized by use of complementary polynucleotide molecules which carry a radioactive or fluorescenic label. Stringency describes the degree of correspondence under certain conditions. The demands with respect to correspondence are higher under high stringent conditions. Under circumstance of hybridization of nucleic acids the stringency conditions are adjusted in dependence of participating nucleic acids as well as use and objective. The conditions for a highly stringent hybridization are such that only very well fitting complementary molecules are able to hybridize. A very well fitting complementary polynucleotide exhibits for example a degree of identity of 90, 91, 92, 93, 94, 95, 96, 97, 98, 99 % with respect to the complementary partner molecule. Under low stringency hybridization occurs also between polynucleotide molecules

which are only complementary within certain segments of the molecule or which have large sections with mismatched or unpaired base pairs.

A hybridization condition of high stringency could be a hybridization wherein the hybridization step in presence of a labeled probe will be carried out in an aqueous 2XSSC solution at 68°C during at least 2 hours, and the following washing steps consist of a first washing in 2XSSC/0,1 % SDS at room temperature for 5 minutes, a second washing in 1 XSSC/0,1 % SDS at 68°C for 1 hour, and a third washing in 0,2 % SSC/0,1 % SDS at 68°C for another hour.

A 2XSSC-, 1XSSC-, or 0,2 XSSC solution is obtained by dilution of a 20XSSC solution. A 20XSSC solution consists of 3 mol/l NaCl and 0,3 mol/l Na-Citrate. The skilled person is well known of other standard methods for hybridization of polynucleotides under stringent conditions. Advice is given to him in particular by textbooks as "Current Protocols in Molecular Biology (Wiley Interscience; ISBN: 0-471-50338-X; eds.: F.M. Ansubel, R. Brant, R.R. Kingston, D.J. Moore, J.G. Seidmann, K. Struhl).

The invention consists further of use of an adenoviral vector consisting of one polynucleotide of the following groups:

a] a polynucleotide having a sequence as specified in SEQ ID NO. 5,

b] a polynucleotide which is 95 % identical to the polynucleotide of SEQ ID NO. 5,

c] a polynucleotide which is at least of the same length as the polynucleotide of SEQ ID NO. 5 and which hybridizes to a polynucleotide of SEQ ID NO. 5 when applying highly stringent hybridization conditions

for constructing of transgenic mammals wherein the G protein coupled receptor EDG2 is transiently or permanently expressed in at least one tissue. Such a tissue is preferably a part of the heart of the mammal. Tissue could also consist of a part of brain, muscle, fat, liver, kidney or other organs of a mammal.

General technical aspects of the invention will be further explained within the following chapters

Adenoviruses can infect a wide variety of cell types and tissues in both dividing and non-dividing cells. This characteristic, together with their relative ease of preparation and purification, has led to their extensive use as gene vectors.
The virus can incorporate only about 2 kb of foreign DNA without significant affects on its stability or its infectivity. The introduction of longer sequences therefore requires the removal of some or all of the virus genes. There are a range of techniques for constructing recombinant adenoviruses.

Vectors can be utilized for (amongst other things): (i) cancer therapy to deliver genes that will lead to tumor suppression and elimination; (ii) gene therapy, i.e. to deliver genes to tissues to augment defective genes; (iii) supplementary therapy to deliver genes, expression of which will combat disease processes.

In the first generation of vectors, the E1 and/or E3 gene cassettes were removed, allowing the introduction of up to 6.5 kb of foreign DNA, often under the control of a heterologous promoter. In the case of the E1 deletions, care was taken to ensure the retention of the ITR and the packaging sequences. Removal of the E1 region had the additional apparent advantage of impairing the transcription of the E2 genes (which are E1 dependent) and consequently the replication of virus DNA and the production of the virus capsid proteins.
The defective E1 viruses could be propagated by infection of 293 cells, which provide the E1 gene products in trans. Although many of the initial studies in vitro provided much promise, it soon became evident that the expression of the transgene in vivo was only transient and was depressed because of the overwhelming immune response, mounted mainly against the virus capsid antigens as well as the expressed transgene. One of the reason for this was the observation that many cells harbored E1-like proteins that allowed the E2 genes to function, albeit at reduced levels. In turn, this facilitated virus DNA replication and the synthesis of the late structural antigens and the production of replication-competent adenovirus (RCA). It also became evident that, at higher m.o.i., the E1 dependence of E2 gene transcription could be ablated.

The next approach was to construct vectors (using suitable complementing cell lines) with som or all of the E2 genes excised and hence with the capacity to replicate virus DNA and to produce RCAs removed. Generation of RCAs could also be prevented by constructing cell lines that do not contain adenovirus sequences that overlap those in the vector. Nevertheless, the host immune response was still a major impediment to achieving persistent transgene expression and was particularly evident when repeated infections were attempted. A number of studies confirmed that the infecting recombinant virus itself was sufficient to induce the immune response, perhaps not surprising in view of the early activation of signaling cascades noted above and the potent antigenicity of the capsid components.

Other, rather more sophisticated vectors (third generation) have been constructed by deleting other virus genes (Amalfitano et al. 1988) and the latest of these have all or nearly all of the virus genes removed. These so-called 'gutless' vectors (Hardy et al., 1997) originally retained only the ITR and packaging sequences and required helper virus and appropriate complementing cells for propagation, followed by careful purification. Nevertheless, there were problems associated with these techniques, mainly due to contaminating helper virus and vector instability. A further development, which prevented the packaging of the helper virus, involved the use of the Cre-lox helper-dependent system.

The AdEasy™ system for the production of recombinant adenoviruses is commercially available from Qbiogene. The construction of a recombinant adenovirus is typically a two-step process in which the desired expression cassette is first assembled into a transfer vector, and subsequently transferred into the adenoviral genome by homologous recombination. Insertion of DNA by homologous recombination is the most efficient way of introducing a gene into an adenovirus vector for two reasons: 1) adenoviral DNAs are large, linear molecules that contain sites for almost all restriction enzymes and 2) the genome is too large (36 kb) to be easily manipulated.

With the AdEasy™ vector system, the backbone vector containing most of the adenoviral genome is used in super coiled plasmid form rather than as linear DNA.

The homologous recombination step is performed in Escherichia coli. In the AdEasy™ system the cDNA of interest is first cloned into a transfer vector. Th resulting plasmid is then linearized with Pme I and co-transformed into E.coli strain BJ5183 together with pAdEasy-1, the viral DNA plasmid. The pAdEasy-1 is E1 and E3 deleted; its E1 functions can be complemented in 293A cells. Recombinations are selected with kanamycin and screened by restriction enzyme analysis. The recombinant adenoviral construct is then cleaved with Pac I to expose its ITR (Inverted Terminal Repeat) and transfected into QBI-293A cells to produce viral particles.

The homologous recombination step is mediated between a linearized transfer vector and an intact super coiled adenovirus plasmid. The kanamycin resistance gene present in the transfer vector allows for the selection of recombinants. Because the cleaved AdEasy™ transfer vectors yield only a low background of kanamycin-resistant colonies, the homologous recombination system has a high signal-to-noise ratio. The E. coli strain BJ5183 is not recA but is deficient in other enzymes that mediate recombination in bacteria and was selected for its higher efficiency of transformation and recombination capabilities. One recombination is achieved and verified, the adenoviral recombinant DNA can simply the transferred to a regular recA, endA strain such as DH5α for greater yields of DNA production. Due to its recA status, DH5α cannot be used to generate adenovirus recombinants by homologous recombination.

Green fluorescent protein (GFP) from Aequora victoria has rapidly become a standard reporter in many biological systems. GFP is unique among light-emitting proteins in that it does not require the presence of cofactors or substrates for the generation of light. In the jellyfish Aequora victoria GFP is acting in a calcium-dependent manner. When Ca^{+2} binds another bioluminescent protein, aequorin, which transfers energy indirectly to GFP to trigger the release of green light. This energy transfer can be mimicked experimentally by exposure of GFP to standard long-wave ultraviolet light. There are GFP isoforms available which emit blue or red light and which are stable at elevated temperatures. GFP was first used to look into living cells by fluorescence microscopy to monitor protein localization and to visualize dynamic cellular events. A fusion between any cloned gene of interest and

GFP can be produced by subcloning techniques and may be introduced into the organism of interest by transient or stable expression. The fate of the resulting protein inside the living cell can th n be followed using conventional fluorescence microscopy. Detection does not require fixation or permenbilization of cells. Likewise a protein may be traced within an animals tissue by simultaneously expressing such protein and GFP.

Providing a cell includes its preparation, cultivation and further processing. Cells are provided, for example, by preparing suitable cell material from organs or tissues or by propagating suitable cell lines or microorganisms. Various suitable culture media can be used for cultivation. The cells are maintained at the optimum temperature for the organism. Where appropriate, preservatives, antibiotics, pH indicators, blood serum components, blood serum, auxiliaries or other substances are added to the growth medium used in each case. Processes for preparation, cultivation and further processing are described in standard textbooks (Example: Basic Cell Culture; Ed. J.M. Davis; IRL Press; 1994).

The application of recombinant techniques provides for a construct, which is to be expressed in a cell, to be present in the form of a polynucleotide sequence which can be prepared by a skilled worker in a routine manner with the aid of his specialist knowledge. The worker skilled in molecular biology / biochemistry can find the specialist knowledge for this, for example, in F.M. Ausubel et al.; Current Protocols in Molecular Biology; John Wiley & Sons; New York. A vector construct is prepared by incorporating a polynucleotide coding for the amino acid sequence of, for example, a GPCR into an expression vector. An expression vector is a vector in which a polynucleotide sequence can be expressed in a host cell into a protein. Vectors may be derived from plasmids, viruses or cosmids and must be capable of autonomous replication. They generally contain an origin of replication, cleavage sites for restriction enzymes and marker genes such as, for example, antibiotic resistance genes. In an expression vector, the polynucleotide sequence which is to be propagated or which has been introduced from the outside is under the functional control of a promoter. A promoter is a functional polynucleotide sequence of variable length, which is used to control transcription, i.e. synthesis of mRNA of a polynucleotide sequence immediately 3'- of said promoter. There are promoters

which are active only in procaryotes, such as, for example, the lac, tac and trc promoters, and also promoters which are active only a eukaryotes, such as, for example, CMV or ADH promoters. In a preferred embodiment, the recombinant vector construct comprises an expression vector usable in eucaryotes and/or procaryotes. An expression vector contains a promoter which can be linked functionally to a polynucleotide sequence so that a protein encoded by said polynucleotide sequence is synthesized in an organism, for example a bacterium, fungus or the cell of a eucaryotic cell line. The promoter may be inducible, by means of tryptophan for example, or may be constitutively active. Examples of expression vectors are pUC18, pUC19, pBluescript, pcDNA3.1 etc.

Transfection is the introduction of foreign polynucleotide sequences into a host cell by means of a vector, and the subsequent propagation of said polynucleotide sequence to any number of identical copies.

A cell line is transiently transfected with a recombinant construct by means of routine methods which can be found by the skilled worker in the abovementioned Current Protocols in Molecular Biology, published by John Wiley & Sons, New York, or in Sambrook et al.; A Laboratory Manual, Cold Spring Harbor Laboratory, ISBN 0-87969-309-6. Examples of such routine methods are electroporation, Ca^{2+}-phosphate coprecipitation and transfection by means of liposomes. The transfected genes may be expressed in the host cell by Western blotting of cell lysates of transfected cells in combination with an immunological detection method. For this too, the required laboratory protocols can be found by the skilled worker in the manuals mentioned above. Specific antibodies for immunodetection of GPCR receptors, which are suitable for carrying out the method of the invention, are commercially available.

A chemical compound is provided in particular by chemical synthesis or isolation of chemical substances from biological material.

The skilled worker may use routine methods for chemical synthesis of a compound or isolation of a substance from cells. Such methods are available to the skilled worker in textbooks such as Organic Synthesis Workbook; 1995; John Wiley & Sons;

ISBN 3-527-30187-9, The Organic Chemistry of Drug Synthesis; 1998; John Wiley & Sons; ISBN 0-471-24510-0, or Bioactive Compounds from Natural Sources; 2001; Taylor & Francis; ISBN 0-7484-0890-8.

The compounds obtained by synthesis or isolation may be dissolved in a suitable solvent. Suitable solvents may contain water, buffer substances (e.g. Tris, HEPES, MOPS, etc.), monovalent and/or divalent ions (e.g. K^+, Na^+, Mg^{2+}, Ca^{2+}, etc.), acids (e.g. HCl, H_2SO_4, formic acid, acetic acid, etc.), bases (e.g. NaOH, etc.), alcohol (e.g. methanol, ethanol, glycerol), detergents (e.g. Na dodecyl sulfate, etc.), organic solvents (e.g. formamide, acetone, dimethyl sulfoxide, etc.) and other components, in particular solubilizers and stabilizers.

The skilled worker can contact the chemical compound with said cell line by using laboratory routine methods. Contacting may take place, for example, in Erlenmeyer vessels, tubes, Eppendorf vessels or on microtiter plates. Temperture-controlled incubators for which a constant temperature of, for example, 30°C or 37°C and fixed CO_2 or humidity conditions can be set may be used for said contacting. Contacting may in particular also be carried out in laboratory robot devices provided therefore (FLIPR). Contacting is possible for different periods of time, from a few seconds to minutes and up to several hours. The conditions to be chosen in each case depend on the receptor, the cell line and the chemical compound.

The final form of a pharmaceutical relates to the final formulation, for example, as tablet, granules, spray, solution, ointment, tincture or other formulation forms.

Processing to the final form refers to the preparation of the particular formulation in generally, the daily dose is in the range from 0.3 mg to 100 mg (typically from 3 mg to 50 mg) per day and per kilogram of body weight, for example, 3-10 mg/kg/day. An intravenous does may be, for example, in the range from 0.3 mg to 1.0 mg/kg and can most suitably be administered as in infusion of from 10 ng to 100 ng per kilogram and per minute. Suitable infusion solutions for these purposes may contain, for example,from 0.1 ng to 10 mg, typically from 1 ng to 10 mg, per milliliter. Single doses may contain, for example, from 1 mg to 10 g of the active substance. It is thus possible for ampoules for injections to contain, for example, from 1 mg to 100 mg,

and for single-dose formulations which can be administered orally, such as, for example, tablets or capsules, to contain, for example, from 1.0 to 1000 mg, typically from 10 to 600 mg.

Examples

The following examples disclose the invention without restricting it to the scope of these examples.

Description of the experimental animals

The animals employed for the studies are ten-week-old female New Zealand white rabbits supplied by Asamhof, Kissing (Germany). At the start of the study the animals have a body weight of between 2.7 and 3.3 kg.

Rearing and housing conditions of the experimental animals

The young are weaned at 30 days. To minimize weaning stress the dam is separated from the young so that the litter initially remains together. After about 10-14 days the young are housed in pairs in fattening cages (l x b x h = 28.5 x 60 x 34 cm) and then after about two weeks they are housed singly. The fatteners are introduced and removed in accordance with the "All in – All out" system to facilitate cleaning and disinfection.

The feed is produced in the in-house agricultural plant and is available to the rabbits ad libitum, as is drinking water.

Climate: The ventilation rate is 10,000 m^3/h in summer and 3000 m^3/h in winter. Particular attention is paid to avoiding drafts. In cold weather the temperature is maintained at about 15°C; overheating of the animal house in summer is prevented as far as possible. Young weaners are kept at about 19°C. Ammonia in the animal

house is to be kept below 30 ppm, relative humidity below 70 %. The breeding house is illuminated for 16 hours at an intensity of about 20 lux.

Study animal housing

The rabbits are brought in an air conditioned vehicle directly to the company, where they are allowed an adaptation period of 2 to 3 days to acclimatize themselves to the new diet and environment.

The rabbits are kept in conventional cages. The cage material consists of stainless steel with PVC inserts; the cage floor has an area of 4040 cm^2 and takes the form of a perforated bottom plate. The feces trays are cleaned daily and the cages are washed and hot-air-sterilized weekly. They are kept under constant conditions at a room temperature between 18 and 21°C and a relative humidity of 55 $\pm$ 5 %. As the animal house has windows the illumination corresponds to the natural night-day cycle with an intensity of at least 100 lux.

Anesthesia and preparation for surgery

On the first day of the study, feed and water are available ad libitum to the rabbits until the start of anesthesia. Then the designated animal is weighed and undergoes a clinical examination, particularly of the cardiovascular system and the respiratory tract. The animals are provided with double intravenous access via indwelling catheters in the left and right lateral auricular veins (Venflon™ 08. x 25 mm), through which anesthesia is then induced with 1 % propofol (Disoprivan, Fresenius AG, Bad Homburg) in a dose of 7 mg/kg body weight i.v. Eye ointment (Vitamin A Dispersa, Ciba Vision®, Grossostheim) is applied to the cornea immediately after induction of anesthesia. After disappearance of the righting reflex the rabbits are shaved ventrally on the neck and on the chest between the elbows and the last rib and are then incubated by advancing a Magill tube with a cuff (internal diameter 2.5 to 3.5 mm, Rüsch AG, Waiblingen) into the trachea during inspiration.

To maintain anesthesia during the operation, the animals are given 2 % propofol (Disoprivan 2 %, Zeneca, Italy) i.v. in a dose of 12 to 14 ml/h via an infusion apparatus (Perfusor®, ED1-300, B. Braun, Melsungen AG). As analgesic the rabbits are given fentanyl (Fentanyl-Janssen 0.5 mg, Janssen-Cilag GmbH, Neuss) in a dose of 0.01 mg/kg i.v. immediately after intubation and then as required during the operation, roughly every 30 minutes, to maintain the surgical tolerance stage.

Using a small-animal ventilator (Anesthesia Workstation, Hallowell EMC, Völker GmbH, Kaltenkirchen), the rabbits are ventilated with 100 % oxygen at a breathing pressure of about 10 mm Hg, a respiratory volume of 8 to 12 ml/kg body weight and a respiratory rate of 29 to 32 breaths per minute, giving a CO_2 partial pressure of about 35 mm Hg in the expired air. Cardiovascular function is monitored intraoperatively via an ECG (Medtronic®, 9790 Programmer, Vitatron Medical B.V., Dieren, Netherlands). Respiration and circulation are monitored by pulse oximetry and capnometry.

Aortic cross-clamping

Preparation of the right common carotid artery. Introduction of a polyurethane catheter (Cavafix®, 1.1* 1.7 mm/16G, ref. 4173589, B. Braun Melsungen AG, Melsungen, Germany) into the artery. After cannulation of the right common carotid artery the chest is opened through the third intercostal space. The virus is introduced into the myocardium using the Sigscreen® method, ensuring that the infused vector remains in that location in particular. The thorax is then closed with ligatures (Nylon®, 2-0 USP) and sutured (Vicryl® (3-0) and Nylon® (3-0)).

Analgesia: The animals receive carprofen (Rimadyl®, Pfizer, 4 mg/kg every 12 hours) and buprenorphine (Temgesic®, Boehringer, 0.01 mg/kg every 12 hours) for 72 hours after the operation.

Euthanasia of the animals

On the last day of the study the animals undergo general anesthesia as describ d in Section 4. Euthanasia takes place in deep narcosis induced by pentobarbital 0.48 g/kg i.v. (Narcoren®, Rhone-Merieux GmbH, Laupheim).

Postmortem macro pathological diagnosis and sampling

Immediately after death the heart is removed as quickly as possible by dividing the chest wall bilaterally at the level of the costal margin and completely disarticulating the sternum. The heart is separated from the afferent and efferent vessels and washed free of blood with cold sterile saline (isotonic sodium chloride solution, Delta-Pharm, Boehringer, Ingelheim) containing 5000 IU heparin. After gross pathological examination and weighing, the heart is preserved intact for further investigation. Hearts intended for single-cell isolation are briefly stored in a sterile tube of cold heparinized saline at about 4°C pending immediate processing. For determination of GFP fluorescence, frozen sections are prepared. Freshly removed hearts intended for cell microscopy are similarly washed with sterile saline, dried with cellulose, and then deep-frozen in a test tube of liquid nitrogen (-196°C) and stored at –80°C until further processing. The animals are autopsied in accordance with veterinary college guidelines, paying particular attention to evaluation of the extent of typical heart failure symptoms: ascites, pleural effusion, heart weight, heart shape, liver congestion and liver weight.

Disposal of the carcasses

After collection in a deep-freeze cabinet at –20°C, the carcasses are fetched by the carcass disposal unit for disposal.

Construction and Purification of Recombinant Adenovirus:

A human EDG2 receptor was cloned by using a PCR-based strategy on the basis of the coding sequence of the human EDG2 receptor. Recombinant (E1/E3-deficient) flag-tagged adenoviruses for this receptor (Ad-EDG2-GFP) were generated,

expressing the transgene and green fluorescence protein (GFP) under control of two independent CMV promoters. As a control, Ad-GFP without further transgenes was used. Large virus stocks were prepared and adenoviral titers were determined using plaque titration and GFP expression titration in non E1-expressing cells.

Cloning of EDG2

The DNA of the EDG2 receptor was amplified from cDNA from human brain by PCR using the forward primer 5'-gcgggggtaccaccatggctgccatctctacttccatcc-3' (SEQ ID NO. 5) and the reverse primer 5'-gcggggctcgagtcacttgtcgtcgtcgtccttatagtcaaccacagagtgatcattgct-3' (SEQ ID NO. 6).

The PCR reaction was performed at 58°C annealing for 1 min and 72°C amplification temperature for 1 min over 20 cycles with the Expand High Fidelity PCR System (Roche Molecular Biochemicals, Mannheim, Germany). Within the PCR reaction, a HA-tag Epitope of 9 amino acids from hemaglutinin of the human influenza A virus) was generated in-frame at the 3'-end of the gene.

The PCR fragment was cloned into the plasmid pAd-Shuttle (Q Biogene, Heidelberg, Germany) by using the restriction sites for KpnI and XhoI and the sequence of resulting pAd Track-CMV-EDG2 was checked by sequencing (MediGenomix, Martinsried, Germany).

SEQ ID NO. 1 discloses the polynucleotide sequence of EDG2 comprising the coding region of a HA-tag within the 30 nucleotides of the 3'-end.

SEQ ID NO. 2 refers to the amino acid sequence of EDG2 comprising the 9 amino acid HA-tag of the C-terminus.

In SEQ ID NO. 8 the polynucleotide sequence of EDG2 having a 5'-HindIII and 3'XhoI site is disclosed. This EDG2 gene has been cloned into HindIII/XbaI sites of pcDNA 3.1 (invitrogen). Such a vector construct is also suitable for amplifying the EDG2 gene.

Construction of recombinant flag-tagged adenovirus (pAD easy 1-EDG2-HA-GFP)

The plasmid pADTrack CMV-EDG2 c-HA was linearized with PmeI (New England Biolabs, Beverly, MA) overnight, dephosphorylated and purified (GFX DNA and Ge1 Purification Kit; Amersham Pharmacia Biotech, Uppsala, Sweden). For homologous recombination, electro competent E. coli BJ5183 (Stratagene, La Jolla, California) were cotransformed with 1 µg of the linearized plasmid pADTrack CMV EDG2 c-HA and 0.1 µg pAdeasyl at 2500 V, 200 W and 25 µFD (E. coli-pulser; Biorad, Heidelberg, Germany), plated and incubated overnight at 37°C. The resulting vector, pAdEasyl-edg2-cHA-GFP, contained the full recombinant adenoviral DNA for Transfection. The full DNA sequence is shown in SEQ ID NO. 5.

The colonies were checked after minipreparation of the plasmid DNA with PacI and the positive clones were retransformed into E. coli DH5a.
For transfection (Effectene Transfection reagent; Qiagen, Hilden, Germany) of 293 cells, plasmid DNA was digested with PacI. The cells were cultured for 7 days and harvested by scraping and centrifugation. The pellet was resuspended in Dulbecco's PBS and the cells were lysed by four repetitive freezing (-80°C) and thawing (37°C) cycles. Cell debris was removed by centrifugation and the lysate stored at –80°C.
For plaque selection of recombinant virus, 293 cells were infected in Dulbecco's PBS for 1 hour at room temperature under gentle agitation with different serial dilutions of lysate from transfection. Following the infection, the cells were overlayed with growth medium containing 0.5 % agarose (1:1 mix of modified Eagles medium 2 x, Gibco Life technologies #21935, supplemented with 20 % Serum, 2x penicillin/streptomycin, 2x L-glutamin and agarose in water 1 %, Seacam). 5-14 days post infection the cell layer was monitored for formation of plaques which were picked using a pasteur pipette, resuspended in 0,5 ml Dulbeccos PBS and stored at –80°C. The plaques were used for further amplification rounds on 293 cells.

Model of Heart Failure

New Zealand White rabbits were treated by rapid pacing at 360 beats/min after pacemaker implantation. Under this protocol, a tachycardia-induced heart failure (HF) develops reproducibly over two weeks. The average +dp/dtmax-value in failing

hearts was 2200±320 mmHg/sec (vs. 3200±390 mmHg/sec in healthy controls; $p<0.05$), and LVEDP increased from 3.6±0.4 mmHg to 13±3.4 ($p<0.05$).

Adenoviral Gene Transfer to Rabbit Myocardium

Before the start of rapid pacing, all rabbits received catheter-based adenoviral gene transfer ($4x10^{10}$ pfu) to the myocardium. For the intervention, the rabbits were anesthetized with fentanyl and propofol. The efficacy of gene transfer was assessed in all hearts after the end of the experiments by investigating transverse freeze-cut sections for expression of GFP by fluorescence microscopy. Morphological changes were assessed after fixation with 4 % paraformaldehyde. Gene transfer led to reproducible transgene expression in ~50 % of cardiomyocytes.

Shortening measurements in isolated cardiomyocytes

Contractility of infected cardiomyocytes was measured by an electro-optical monitoring system connected to online digitalized assessment of amplitude and velocity of shortening and of relaxation. Transgene-positive cardiomyocytes were identified by co-expression of GFP under fluorescent light. After the contraction amplitude reached stability, increasing concentrations of isoproterenol were applied at constant concentrations of lysophosphatidic acid (LPA; 10^{-5} mol/l); or increasing LPA concentrations were added to constant concentrations of isoproterenol (10^{-8} mol/l).

Single cell contraction

In order to investigate the effects of EDG2 on cardiomyocyte contractility, fractional shortening and velocity of shortening in single, isolated cardiomyocytes from failing hearts after ex vivo gene transfer was measured. At a concentration of lysophosphatidic acid (LPA) of 10^{-5} mol/l which does not alter basal contractility, increasing concentrations of isoproterenol had a significantly lower positively inotropic effect in EDG2-overexpressing cardiomyocytes (Fig. 1). After prestimulation with a low concentration of isoproterenol (10^{-8} mol/l), increasing concentrations of LPA showed a significant negatively inotropic effect in EDG2-overexpressing

cardiomyocytes whereas no effect was observed in the control GFP group (Fig. 2). In the absence of prestimulation with isoproterenol, LPA has no effect on th contractility of cardiomyocytes.

Western blot of infected cardiomyocytes

Cardiomyocytes were harvested 48 hours after adenoviral infection. The cells were homogenized and cytosolic extracts were then used for western blotting with antibodies against the HA tag or against EDG2. Horse radish peroxidase-coupled goat anti-rabbit antibodies by Dianova, Germany, were used as second antibodies.

In vivo adenoviral delivery of transgene to failing heart

Overexpression of all transgenes was investigated by studying the co-expression of GFP in the hearts after in vivo gene transfer, since all transgenes were expressed together with GFP. A macroscopic slice of a rabbit heart infected with Ad-EDG 2-GFP showed GFP co-expression occurring throughout the left ventricle when determined by anti-GFP antibody staining.

Transgene expression assessed by Western blotting

Western blotting documented the expression of EDG2 by means of an antibody directed against the HA tag or by a specific antibody against EDG2 in cardiomyocytes.

Preparation and culture of adult ventricular cardiomyocytes and adenovirus infections

Single calcium-tolerant ventricular cardiomyocytes were isolated from failing White New Zealand rabbit hearts. Briefly, the hearts were perfused and digested with collagenase. The isolated cardiomyocytes were cultured in modified M199 on laminin-precoated dishes (5-10 $\mu g/cm^2$) at a density of $1.5x10^5$ cells per cm^2 (at 5 % CO_2 and 37°C). For contraction experiments, the cells were infected with adenovirus (multiplicity of infection (moi) 1 pfu/cell) 5 hours after plating. 50-60 % of the infected cardiomyocytes expressed the transgene at this titer.

Myocardial Contractility Measurement by Echocardiography and Intraventricular Tip Catheter

Left ventricular contractility was examined by echocardiography before the initiation of rapid pacing, after 1 week and after two weeks after the start of pacing. Tip catheter measurements were performed after 2 weeks of pacing. The rabbits were anesthetized; ECG was monitored continuously.

For echocardiography, a 7.5 MHz probe was fixed on a tripod. Standard sections were recorded, which were well reproducible. For tip catheter measurements, a Millar 3F tip catheter connected to a differentiating device was placed in the left ventricle via a sheath placed in the carotid artery. After definition of basal contractility and left ventricular pressure, 200 µL of NaCl (0.9 %) was injected as a negative control. Isoproterenol and lysophosphatidic acid (LPA) were infused intravenously at increasing doses. After a 20 min equilibration period, tip catheter measurements were carried out.

Deterioration of LV dysfunction in pacing-induced heart failure

Fig. 3 shows tip catheterization measurements after 2 weeks of rapid pacing in rabbits suffering from severe heart failure (NYHA IV). In the EDG2-expression group, the first derivatives of LV pressure (dp/dt max) were significantly lower than in the Ad-GFP-infected control group at basal conditions and at increasing doses of LPA. This was also true for the increases in systolic LV pressure (Fig. 4).

Echocardiography showed a marked hypertrophy of the myocardium after 2 weeks in the EDG2-overexpressing hearts, which was also evidenced by decreases in systolic and diastolic diameters. The mean thicknesses of the posterior wall and of the septum of the LV were significantly greater in EDG2-overexpressing hearts compared to the GFP controls. The time course of LV fractional shortening (FS) was assessed by serial echocardiography during the two week-observation period. In both groups, FS declined gradually during the time period of rapid pacing.

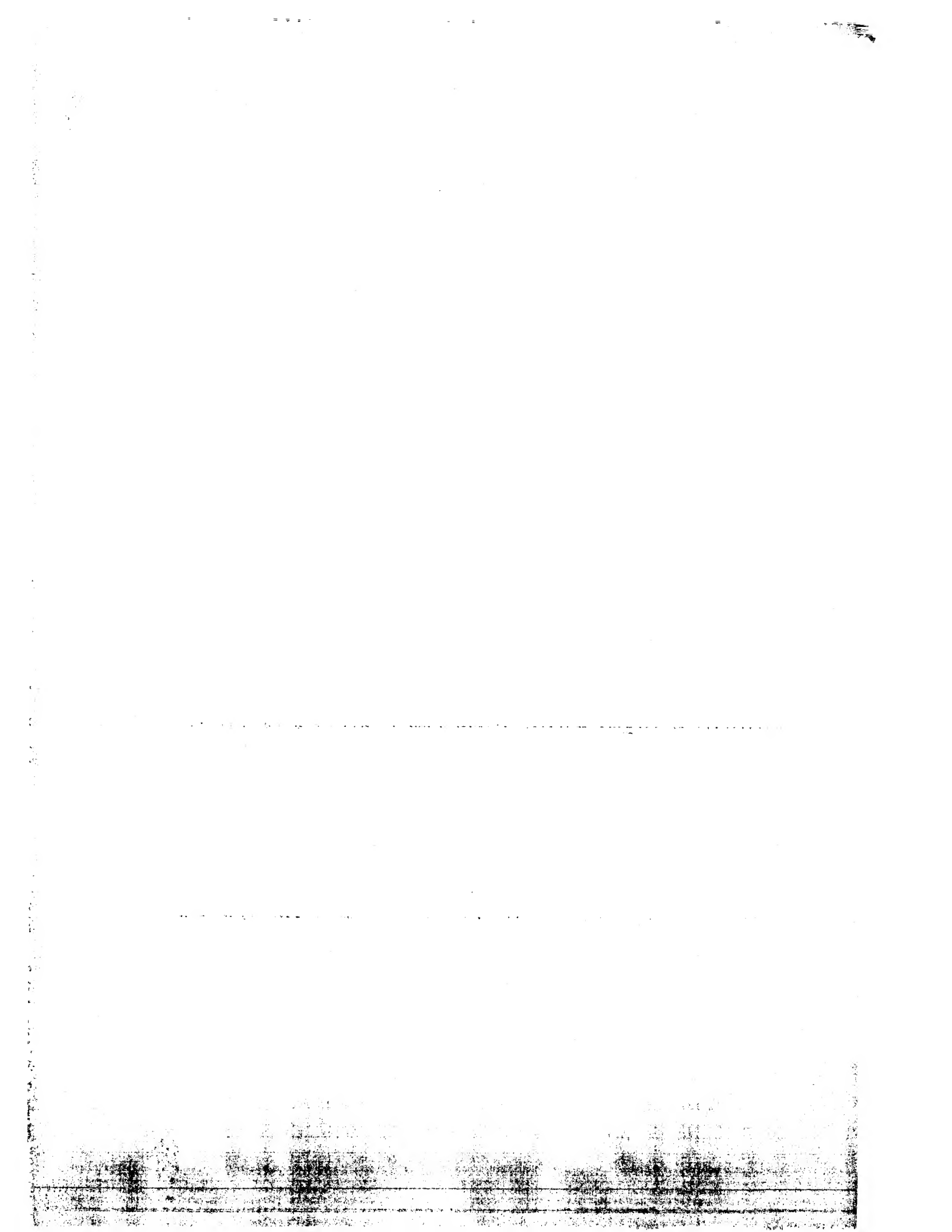
Description of Figures:

Fig. 1:
Contraction amplitude of single cardiomyocytes isolated from failing hearts. The cardiomyocytes were infected ex vivo with either Ad-GFP or Ad-EDG2-GFP. Fractional shortening (FS) was determined in response to increasing concentrations of isoproterenol after prestimulation with 10 µM of LPA. Data represent means ± SEM.

Fig. 2:
Contraction amplitude of single cardiomyocytes isolated from failing hearts. Similar to the experiments shown in Figure 1, FS was compared in cardiomyocytes after gene transfer with either Ad-GFP or Ad-EDG2-GFP in vitro. Fractional shortening was determined in response to increasing LPA concentrations after prestimulation with 10 µM of isoproterenol. Data represent means ± SEM.

Fig. 3:
Maximum first derivative of left ventricular pressure (LV dp/dt max) at baseline and in response to increasing doses of LPA as determined by tip catheterization. In rabbits with terminal heart failure due to rapid pacing and after two weeks after gene transfer of either GFP or EDG2. Data represent means ± SEM. All measurements were done in 8 animals in triplicates *$p<0.05$ vs GFP.

Fig. 4:
Left ventricular systolic pressure at baseline and in response to increasing doses of LPA as determined by tip catheterization in rabbits with terminal heart failure due to rapid pacing and after two weeks after gene transfer of either GFP or EDG2. Data represent means ± SEM. All measurements were done in 8 animals in triplicates. *$p<0.05$ vs GFP.



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Claims

Claim 1

Myocardial cell of a mammal which cell contains an adenoviral vector sequence for simultaneous expression of G protein coupled receptor EDG2 and GFP.

Claim 2

Myocardial cell of a mammal as claimed in claim 1 wherein the adenoviral vector sequence consists of a recombinant E1/E3 deficient adenovirus which expresses the G protein coupled receptor EDG2 and GFP under control of two independent promoters.

Claim 3

Myocardial cell of a mammal as claimed in claim 2 wherein the two independent promoters are two CMV promoters.

Claim 4

Myocardial cell of a mammal as claimed in one of claims 1 to 3 which contains protein of G protein coupled receptor EDG2 and protein of GFP.

Claim 5

Myocardial cell of a mammal as claimed in one of claims 1 to 4 wherein the mammal is a rabbit, mouse or rat.

Claim 6

Production of a myocardial cell as claimed in claims 1 to 5 wherein

a] the heart of a mammal is removed by state of a veterinary medicine operative techniques,

b] the heart is perfused and digested with collagenase,

c] the isolated cardiomyocytes are infected with an adenoviral vector consisting of a recombinant E1/E3 deficient adenovirus which expresses the G protein coupled receptor EDG2 and GFP under control of two independent promoters.

Claim 7

Mammal having a myocardium which contains an adenoviral vector for simultaneous expression of a G protein coupled receptor EDG2 and GFP.

Claim 8

Mammal as claimed in claim 7 wherein the adenoviral vector sequence consists of a recombinant E1/E3 deficient adenovirus which expresses the G protein coupled receptor EDG2 and GFP under control of two independent promoters.

Claim 9

Mammal as claimed in claim 8 wherein the two independent promoters are two CMV promoters.

Claim 10

Mammal as claimed in one of claims 7 to 9 which myocardium contains protein of G protein coupled receptor EDG2 and protein of GFP.

Claim 11

Mammal as claimed in one of claims 7 to 10 being a rabbit, a mouse, or a rat.

Claim 12

Production of a mammal as claimed in claims 7 to 11 wherein

a] an adenoviral vector sequence for simultaneous expression of G protein coupled receptor EDG2 and GFP is provided,

b] a mammal is provided,

c] the adenoviral vector sequence from a] is transferred into the myocardium of the mammal from b] by means of a catheter.

Claim 13

Use of a mammal of claims for producing a myocardial cell as claimed in claims 1 to 6 for performing of claims 14 and 15.

Claim 14

Method for identification of a compound, which modifies the activity of G protein coupled receptor EDG2, wherein

a] a transformed cell from a heart muscle which expresses the receptor EDG2 or a fusion protein comprising the receptor EDG2 is provided,

b] possibly a treatment of the cell from a] is performed by use of isoproterenol and/or lysophosphatidic acid,

c] a chemical compound is provided,

d] the cell from a] or b] is brought in contact with the chemical compound from c],

e] the contractility of a cell from d] is determined and is brought in relation to contractility of a cell which has the same characteristics as a cell from a] but has not brought in contact with a chemical compound from c] wherein a relative enhancement or reduction of contractility of the cell which has brought in contact with a chemical compound according to d] by this compound demonstrates the ability of such compound to modify the activity of receptor EDG2.

Claim 15

Method for identification of a compound which modifies the activity of G protein coupled receptor EDG2, wherein

a] a transformed cell from a heart muscle which expresses the receptor EDG2 or a fusion protein comprising the receptor EDG2 is provided,

b] possibly a treatment of the cell from a] is performed by use of isoproterenol and/or lysophosphatidic acid,

c] a chemical compound is provided,

d] the cell from a] or b] us brought in contact with the chemical compound from c],

e] the contractility of a cell from d] is determined and is brought in relation to contractility of a cell of same cell type as a cell according to a] but which does not express a receptor EDG2 or a fusion protein comprising a receptor EDG2 wherein a relative enhancement or reduction of contractility of the cell which expresses a receptor EDG2 or a fusion protein comprising a receptor EDG2 by a compound demonstrates the ability of such compound to modify the activity of receptor EDG2.

Claim 16

Recombinant adenoviral vector consisting of one polynucleotide of the following groups:

a] a polynucleotide having a sequence as specified in SEQ ID NO. 5,

b] a polynucleotide, which is 95 % identical to the polynucleotide of SEQ ID NO. 5,

c] a polynucleotide, which is at least of the same length as the polynucleotide of SEQ ID NO. 5 and which hybridizes to a polynucleotide of SEQ ID NO. 5 when applying highly stringent hybridization conditions.

Claim 17

Recombinant adenoviral vector as claimed in claim 16 comprising a polynucleotide sequence which is encoding a protein of SEQ ID NO. 2.

Claim 18

Use of an adenoviral vector of claims 16 and 17 for constructing of transgenic mammals wherein the G protein coupled receptor EDG2 is transiently or permanently expressed in at least one tissue.

Claim 19

Use of an adenoviral vector of claim 18, wherein the tissue is part of the heart.

Summary

The invention refers to a transient transformed mammal which is useful as animal model for heart failure.

Fig. 1

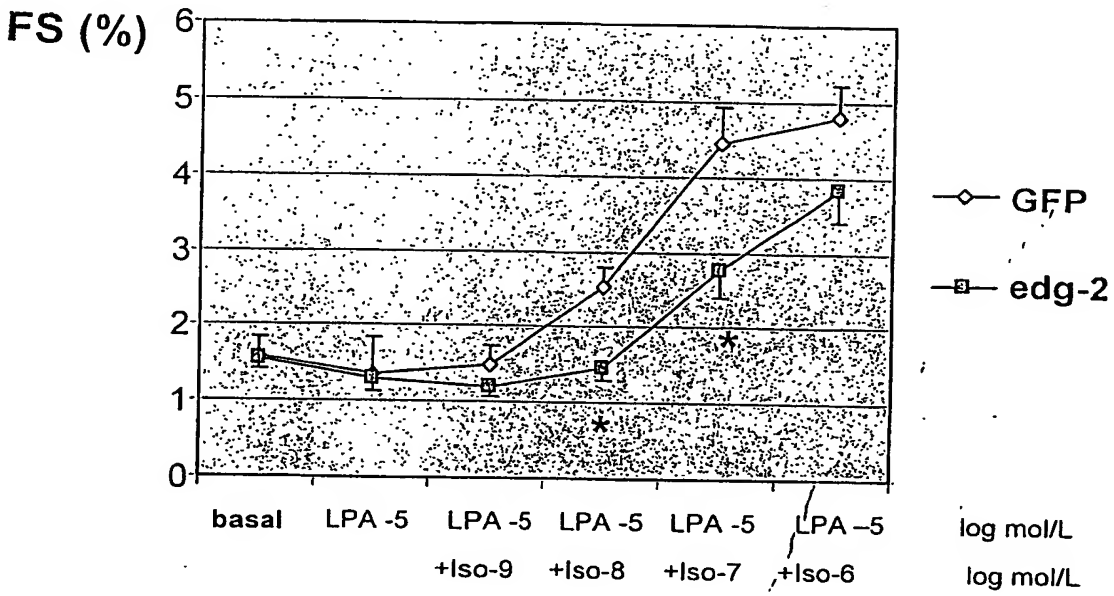
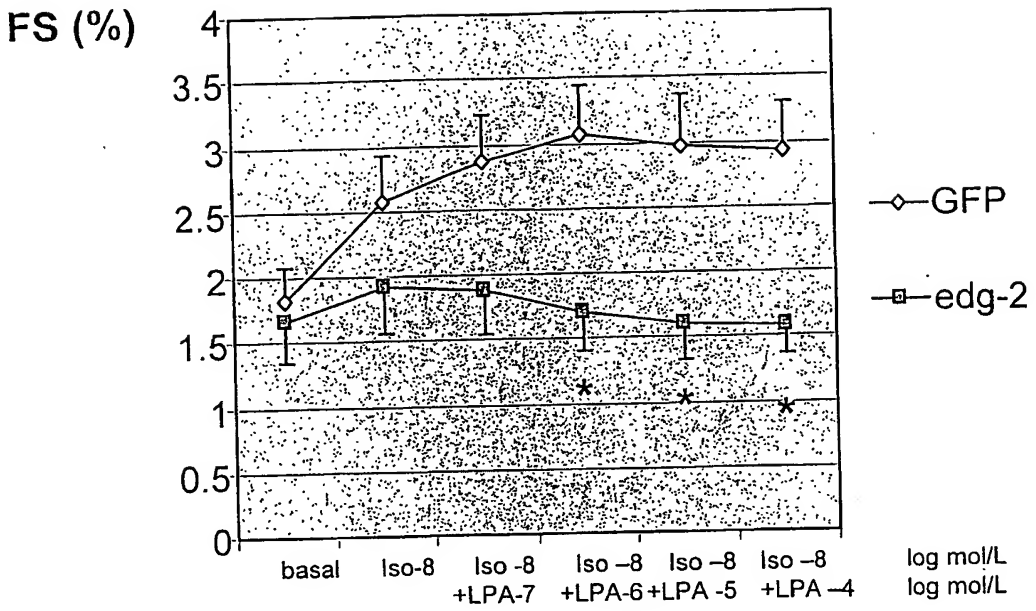


Fig. 2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3
2.5
2
1.5
1
0.5
0
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Iso –8 +LPA-6
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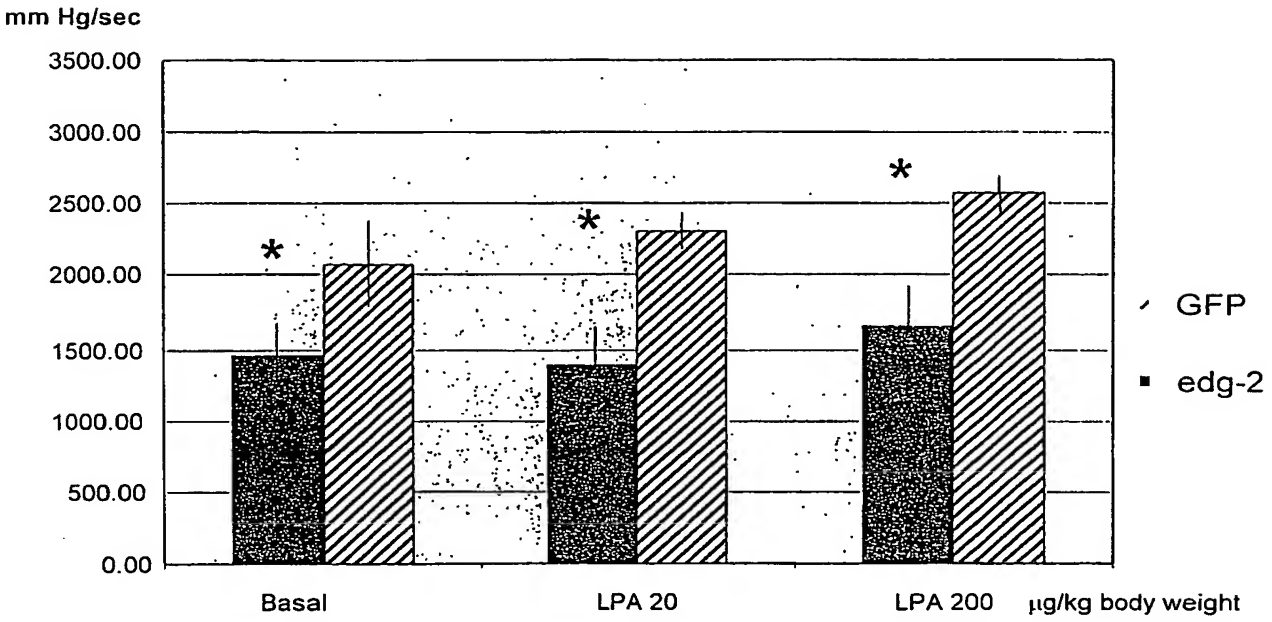
log mol/L
log mol/L
GFP
edg-2
*
*
*

Fig. 3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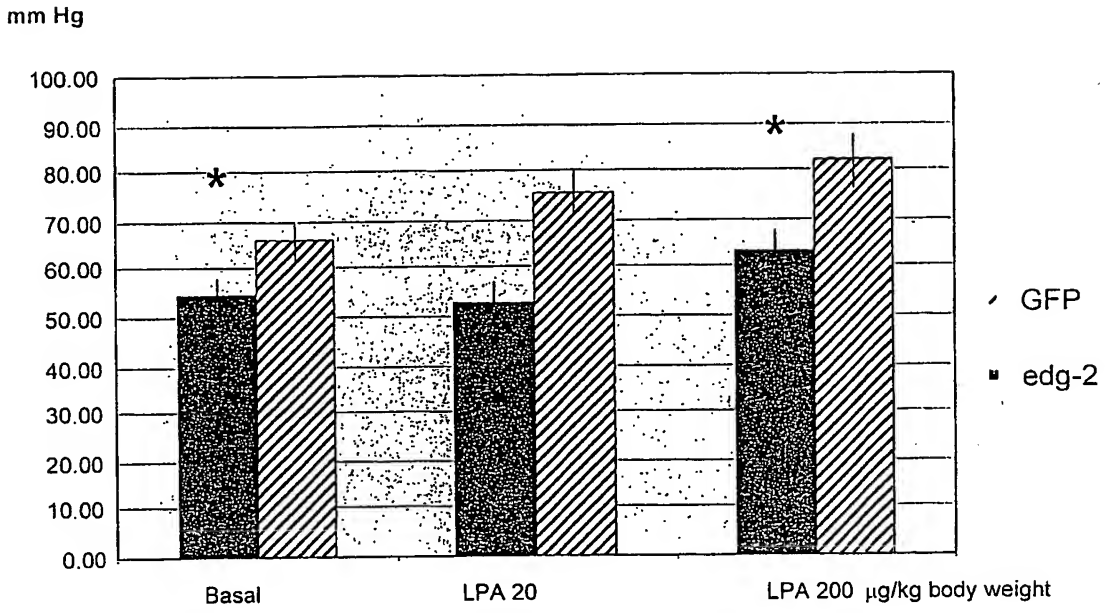
μg/kg body weight

Fig. 4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